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Production of Vanillic Acid from Vanillin by Resting Cells of *Serratia marcescens*

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Resting-cell suspensions of *Serratia marcescens* were able to convert, quantitatively, 0.3% vanillin to vanillic acid. The vanillic acid-producing activity reached a maximum after 28 h of incubation with 0.01% vanillin as an inducer.

There is increasing interest in the use of whole-cell bioconversion processes in the synthesis of fine chemicals (4, 15, 16). Vanillin oxidation to vanillic acid by microorganisms has been known for many years (6), and recently this oxidation by whole-cell suspensions of *Streptomyces viridosporus* and *Aspergillus japonicus* has been reported (10, 13). We report here such an oxidation with resting-cell suspensions of *Serratia marcescens*. The strain, isolated from a compost pile, was identified according to *Bergey's Manual of Systematic Bacteriology* (9) and showed positive results for the following characteristics: motility, prodigiosin production, catalase, Voges-Proskauer test, β-galactosidase, hydrolysis of gelatin and esculin, lysine and ornithine decarboxylases, and growth at 40°C. The strain was negative for the following characteristics: Gram strain, phenylalanine and tryptophan deaminases, arginine decarboxylase, methyl red test, and indole production.

Cultivation parameters were determined in 250-ml Erlenmeyer flasks with 100 ml of mineral medium (12) supplemented with glucose, vanillin, or both. The cultures were incubated on a reciprocal shaker (Kottermann; 120 strokes per min) at 28°C for 24 h, except when specified otherwise. The cell yields were determined with a Bausch & Lomb Spectronic 20 spectrophotometer by measuring the optical density at 600 nm and by viable cell counts on nutrient agar (Difco Laboratories) plates.

The vanillate-producing activity was determined by growing *S. marcescens* in a medium containing 0.1% glucose in the absence or presence of vanillin (0.65 mM). Samples were removed from cultures after 12 to 52 h of incubation, and cell extracts were obtained after sonic treatment (60 W, 7 min, 0°C; Sonifier B-12; Branson Sonic Power Co). Then 350 μl of the enzyme extract was added to 1,500 μl of Na-K phosphate buffer (100 mM, pH 7) containing vanillin (0.33 mM), and the decrease in the aldehyde A_{345} maximum was followed for 30 to 40 min. A control experiment with boiled (5 min) enzyme extract was carried out. One unit of enzyme activity was defined as the amount of enzyme oxydizing 1 μmol of substrate ml^{-1} min^{-1}. The specific activities of proteins were determined by the method of Bradford (5).

The bioconversion experiments for vanillin (0.1 to 0.4%, wt/vol) and vanillic acid (0.01%, wt/vol) were carried out with resting-cell suspensions (10^8 viable cells per ml) in Na-K phosphate buffer (100 mM, pH 7; 200 ml in a 1-liter Erlenmeyer flask). The conversion media were reciprocally shaken at 28°C. Samples, taken out at intervals, were analyzed by silica gel thin-layer chromatography (hexane-ethyl acetate, 1:1) and by UV-visible light spectrophotometry (Hitachi 100-80 spectrophotometer). The bioconversion media, where only the conversion product was detected, were acidified to pH 2 to 3 (20% H_2SO_4) and treated with ethyl acetate (five times with 100 ml). The acidic compound was extracted in saturated $NaHCO_3$ solution (five

TABLE 1. Effect of glucose concentration on viable cell production of *S. marcescens* after 24 h of growth at 28°C in liquid mineral medium

Glucose concn (%, wt/vol)	Viable cells per ml, 10^8
0.06	2.4
0.08	3.0
0.1	7.1
0.3	7.1
0.5	6.3

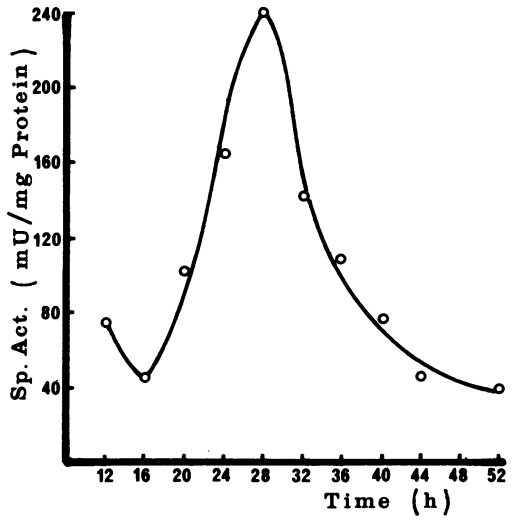

FIG. 1. Time course of vanillic acid-producing activity. Culture was carried out in the presence of glucose (0.1%) and vanillin (0.01%).

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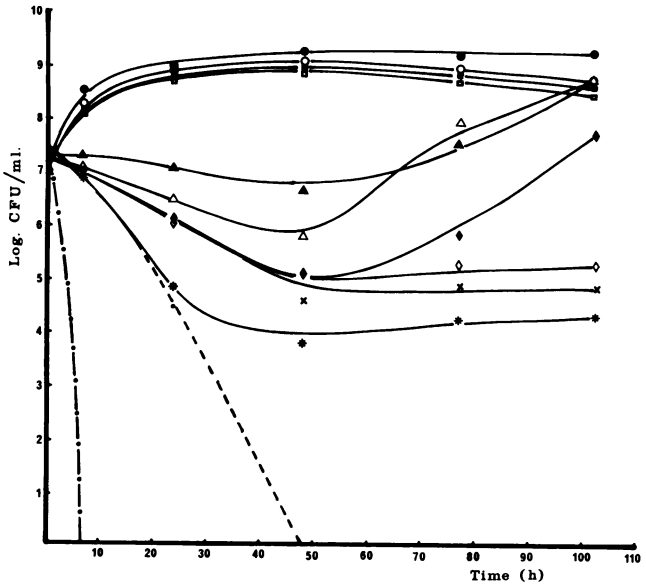

FIG. 2. Effect of different vanillin concentrations on growth of *S. marcescens*. All cultures were supplemented with glucose (0.1%). Symbols (vanillin concentrations): ●, control without vanillin; ○, 0.1%; ■, 0.15%; □, 0.2%; ▲, 0.25%; △, 0.3%; ◆, 0.35%; ◇, 0.4%; ×, 0.45%; *, 0.5%; ---, 0.55%; ·-·-, 1%.

times with 100 ml) from the organic layer. This alkaline solution was acidified to pH 2 to 3 and extracted with ethyl acetate (five times with 100 ml), and the solvent was removed under vacuum to give the bioconversion product. The compound was crystallized from hexane-ethyl acetate, and the melting point was determined on a Reichert Thermovar apparatus. Electron impact mass spectra were obtained on a VG Micromass ZAB-2F (70 eV) instrument, and ^1H nuclear magnetic resonance spectra were achieved on a Bruker WP-2000 SY spectrometer in $CDCl_3$.

No significant increase in cell yields was obtained with up to 0.1% glucose (Table 1). The vanillic acid-producing activity reached a maximum (0.24 μmol of oxidized substrate min^{-1} mg of $protein^{-1}$) after 28 h of incubation with 0.01% vanillin as the inducer (Fig. 1). After 2 h, in the same culture conditions, vanillin was completely converted, although the bacterial growth was in the lag phase. When no vanillin was added the specific activity was 4.2-fold lower. The vanillin concentration had a complex effect on cell growth (Fig. 2). At concentrations between 0.1 and 0.25% it allowed growth; between 0.25 and 0.35% vanillin first acted as a bactericide, allowing growth later; between 0.4 and 0.5% it was first bactericidal and later bacteriostatic; and between 0.55 and 1% it was bactericidal only.

According to the above-mentioned results the biomass for the bioconversion of vanillin to vanillic acid was obtained after incubation of *S. marcescens* (28 h) in a medium with

TABLE 2. Effect of vanillin concentration on vanillic acid production with resting cells

Vanillin (mg ml^{-1})	Time (h)	Vanillic acid (mg ml^{-1})	Conversion (%)
1	6	1.1	100
2	20	2.16	99
3	96	3.3	100
4	168	2.2	50

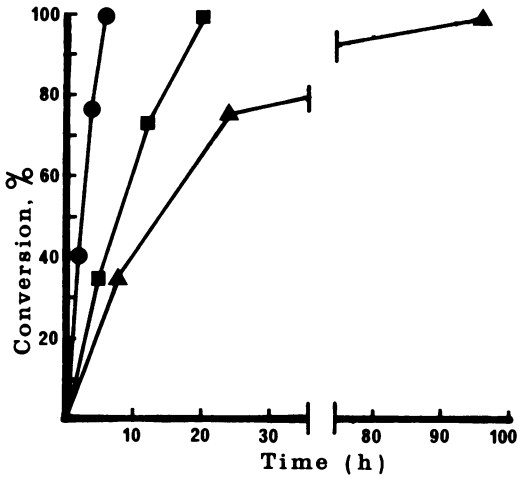

FIG. 3. Conversion of vanillin to vanillic acid by resting cells of *S. marcescens* at different vanillin concentrations. Symbols (vanillin concentrations): ●, 0.1%; ■, 0.2%; ▲, 0.3%.

0.1% glucose and 0.01% vanillin. Vanillin concentrations of 0.1, 0.2, and 0.3% were quantitatively transformed to vanillic acid, in the bioconversion media, after 6, 20, and 96 h, respectively (Fig. 3, Table 2). Also, thin-layer chromatography confirmed the complete conversion. At 0.4% vanillin a 50% conversion was observed after a 168-h incubation period. The pHs of the media did not change over those time periods in the concurrent controls, vanillin remained unchanged, and vanillate (0.01%) was not transformed.

Recoveries of 100, 219, and 331 mg of vanillic acid per 100 ml of bioconversion medium were obtained from media containing 100, 200, and 300 mg of vanillin, respectively. The isolated vanillic acid had a melting point of 196 to 197°C and was identified by comparison of its physical and spectral data with those of an authentic sample (melting point, mass spectrometry, and ^1H nuclear magnetic resonance).

The data in Fig. 3 also roughly point out that until 75% conversion the transformation rate decreased in proportion to the vanillin concentration, which could be attributed to a bactericidal effect of vanillin (1, 3, 11). At 0.3% vanillin a considerable decrease in the rate was observed at a conversion greater than 75%. It was probably caused mainly by a vanillic acid bactericidal effect.

As far as we know this is the first report of a rapid and complete oxidation of vanillin to vanillic acid at a high substrate concentration (0.3%) by resting-cell suspensions, and the process may be commercially useful (2, 7, 8, 14). Work is in progress to extend this transformation by *S. marcescens* to several other aromatic and aliphatic aldehydes, since the relaxed specificity of the involved enzymes should permit such an oxidation (6, 13, 15).

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LITERATURE CITED

1. **Ander, P., A. Hatakka, and K. E. Eriksson.** 1980. Vanillic acid metabolism by the white-rod fungus *Sporotrichum pulverulentum*. Arch. Microbiol. **125**:189–202.
2. **Bock, L. H., and J. K. Anderson.** 1955. Linear polyesters derived from vanillic acid. J. Polym. Sci. **17**:553–558.
3. **Borneman, W. S., D. E. Akin, and W. P. Van Eseltine.** 1986. Effect of phenolic monomers on ruminal bacteria. Appl. Envi-

ron. Microbiol. **52:**1331–1339.
4. **Bowen, W. R., N. Lambert, S. Y. R. Pugh, and F. Taylor.** 1986. The yeast alcohol dehydrogenase catalysed conversion of cinnamaldehyde to cinnamyl alcohol. J. Chem. Technol. Biotechnol. **36:**267–272.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Crawford, D. L., J. B. Sutherland, A. L. Pometto III, and J. M. Miller.** 1982. Production of an aromatic aldehyde oxidase by *Streptomyces viridosporus*. Arch. Microbiol. **131:**351–355.
7. **Era, V., and J. Hannula.** 1974. Polyester from vanillin: synthesis and characterization. Pap. Puu **5:**489–496.
8. **Galloway, D.** 1975. Growing role of wood products in the livestock feeding field. Pulp Pap. **49:**104–105.
9. **Grimont, P. A. D., and F. Grimont.** 1984. The genus *Serratia*, p. 477–484. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
10. **Milstein, O., Y. Vered, L. Shragina, J. Gressel, H. M. Flowers, and A. Huttermann.** 1983. Metabolism of lignin related aromatic compounds by *Aspergillus japonicus*. Arch. Microbiol. **135:** 147–154.
11. **Nishikawa, N. K., R. Sutcliffe, and J. N. Saddler.** 1988. The influence of lignin degradation products on xylose fermentation by *Klebsiella pneumoniae*. Appl. Microbiol. Biotechnol. **27:** 549–552.
12. **Odier, E., and B. Monties.** 1978. Biodegradation de la lignine de blé par *Xanthomonas* 23. Ann. Microbiol. (Paris) **129A:**361–377.
13. **Pometto, A. L., III, and D. L. Crawford.** 1983. Whole-cell bioconversion of vanillin and vanillic acid by *Streptomyces viridosporus*. Appl. Environ. Microbiol. **45:**1582–1585.
14. **Rai, R. P., and M. S. Maurya.** 1966. Synthesis and evaluation of antibacterial activity of vanillin derivatives. J. Sci. Technol. (Peshawar) **4:**275–276.
15. **Spain, J. C., and D. T. Gibson.** 1988. Oxidation of substituted phenols by *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS6. Appl. Environ. Microbiol. **54:**1399–1404.
16. **Yamada, H., and S. Shimizu.** 1988. Microbial and enzymatic processes for the production of biologically and chemically useful compounds. Angew. Chem. Int. Ed. Engl. **27:**622–642.